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TITLE: Development of an Autologous Macrophage-based Adoptive Gene Transfer Strategy to Treat Posttraumatic Osteoarthritis (PTOA) and Osteoarithritis (OA)

PRINCIPAL INVESTIGATOR: Matilda Sheng, Ph.D.

CONTRACTING ORGANIZATION: Loma Linda University
Loma Linda, CA 92350

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Matilda Sheng, Ph.D.

e-mail: msheng@llu.edu

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Loma Linda University 11060 Anderson St. BLDG Magan Hall

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14. ABSTRACT

OA is the most common degenerative joint disease, and ~12% of all OA are resulted from an acute trauma to the joint and are referred to as PTOA. There is no cure for PTOA or OA. This Discovery Award project seeks to obtain proof-of-concept type of evidence for the feasibility of and efficacy for an innovative autologous macrophage-based anti-catabolic and pro-chondrogenic combination adoptive gene therapy for treatment of PTOA. The rationale for the use of macrophages as the cell vehicle for targeted delivery and confined expression of the transgene(s) is based on definitive evidence that a) PTOA development is associated with both acute and chronic inflammation of the synovium; and b) synovial inflammation triggers massive infiltration of activated macrophages. The idea of the combination macrophage-based adoptive gene therapy with both an anti-catabolic gene (IL-1ra or IL-1β shRNA) and a pro-chondrogenic gene (TGFβ3) is based on the assumption that comprehensive treatment of a disease with complex pathophysiology, such as PTOA, will require concerted treatments at multiple phases of the diseases. The proposed study will test two hypotheses: 1) *the autologous macrophage-based adoptive gene transfer strategy can effectively deliver and confine expression of an anti-catabolic gene (IL-1ra or IL-1β shRNA) along with a chondrogenic gene (TGFβ3) in the inflamed areas within the synovium of the PTOA joint*; and 2) *the IL-1ra (or IL-1β shRNA) and TGFβ3 combination autologous macrophage-based adoptive gene transfer strategy will reduce PTOA symptoms and promote articular cartilage regeneration in a mouse PTOA model*. Aim 1 will show that: 1) intra-articular injection of donor macrophages will lead to confined and long-term recruitment of donor cells at the inflamed surface of the injured articular cartilage; 2) C57BL/6J macrophages can be effectively transfected by lentiviral vectors to express large amounts of transgene; and 3) intra-articular injection of genetically modified syngenic mouse macrophages into the PTOA synovium will result in confined and sustained production of large amounts of the transgenes (i.e., IL-1ra and TGFβ3) at and around the surface of the inflamed articular cartilage. Aim 2 will test the dose- and time-dependent effects of the macrophage-based IL-1ra/TGFβ3 combination adoptive gene therapy on the suppression the inflammation-induced degradation of articular cartilage (and/or erosion of endochondral bone) and on the stimulation of the TGFβ3-mediated chondrogenesis to repair and regenerate the damaged articular cartilage, respectively. This work will be performed in a mouse intra-articular tibial fracture PTOA model (with C57BL/6J mice).

15. SUBJECT TERMS nothing listed

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I. Introduction:

Osteoarthritis (OA) is the most common degenerative joint disease, and ~12% of all OA are resulted from an acute trauma to the joint and are referred to as PTOA [1]. It is estimated that the total cost of OA cases is close to $100 billion, of which nearly 50% is from lost earnings [2, 3]. Military personnel have a significantly greater incidence of PTOA than the general population of the same age range [4], which is likely due to the intense physical demands of military-related activities or combat-related traumatic joint injuries. Either direct joint damage or limb amputation result in increasing loads on contralateral joint surfaces, gradually leads to the development of PTOA. There is no cure for PTOA or OA. Treatment options vary, including physical therapy, lifestyle changes, orthopedic bracing, and medications. Joint replacement surgery may also be required in eroding forms of arthritis. Medications can help reduce inflammation in the joint to reduce pain, and slow the progress of joint damage [5]. This project seeks to obtain proof-of-concept type of evidence for the feasibility of and efficacy for an innovative autologous macrophage-based anti-catabolic and pro-chondrogenic combination adoptive gene therapy for treatment of PTOA, and tests two specific hypotheses: 1) *the autologous macrophage-based adoptive gene transfer strategy can effectively deliver and confine expression of an anti-catabolic gene (IL-1ra or IL-1β shRNA) along with a chondrogenic gene (TGFβ3) in the inflamed areas within the synovium of the PTOA joint*; and 2) *the IL-1ra (or IL-1β shRNA) and TGFβ3 combination autologous macrophage-based adoptive gene transfer strategy will reduce PTOA symptoms and promote articular cartilage regeneration in a mouse PTOA model*. If successful, this therapy will provide an innovative, safe, cost-efficient and non-invasive alternative for treatment of PTOA and other forms of arthritis. Thus, this proposal has very high clinical significance to the military, veterans, and civilian populations.

II. Keywords:

Osteoarthritis; Post-traumatic; IL1ra; TGF-β3; Transduction, Adoptive therapy, Macrophages, Chondrocytes; Articular Cartilage; Regeneration; Mice

III. Overall Project Summary:

The primary objective of this proposal during the reporting period is to obtain proof-of-concept evidence for the prevention and treatment using this novel macrophage-based adoptive gene transfer approach. This project has two Specific Aims:

Aim 1. To demonstrate that the macrophage-based adoptive gene transfer strategy can effectively deliver and yield confined, sustained expression of transgenes in the inflamed synovia of the PTOA.

Aim 2. To demonstrate that the macrophage-based IL-1ra (or IL-1β shRNA) and TGF-β3 combination adoptive gene therapy can effectively treat and prevent PTOA in a mouse PTOA model.
These two Aims will be accomplished through the following four Technical Objectives (milestones):

Milestone 1 (Technical Objective 1):*To demonstration that the macrophage-based adoptive gene transfer strategy can effectively deliver and yield confined expression of transgenes in the inflamed synovium of the PTOA* (Aim 1).

Specific Task 1 is to establish the closed intra-articular tibial plateau fracture PTOA model in the knee of C57BL/6J mice for use in subsequent work - *This specific task is 100% completed.*

Specific Task 2 is to demonstrate that the injected donor GFP-expressing M2 macrophages are recruited to and retained in the inflamed articular cartilage surface in the synovium of the PTOA knee joint for substantial period of time after intra-articular injection (Aim 1a) - *This specific task is 100% completed.*

Milestone 2 (Technical Objective 2): *To demonstrate that primary mouse macrophages can be effectively transduced with lentiviral vectors and express substantial amounts of the transgenes (i.e., IL-1ra or TGFβ3).*

Specific Task 1 is to construct the required lenti-viral based vectors that can effectively express murine Il-1ra, TGF-β3, or GFP marker genes- ***This specific task is 100% completed and our progress has been summarized in the previous annual report****.*

Specific Task 2 is to confirm that the isolated mouse macrophages can be effectively transduced by the lenti-viral vectors expressing IL-1ra, TGFβ3, or GFP *ex vivo*, and to confirm that the transduced cells express substantial amounts of the transgene (Aim 1b) – ***This specific aim is 100% completed and our progress has been summarized in the previous annual report****.*

Milestone 3 (Technical Objective 3). *To develop the macrophage-based adoptive therapy expressing IL-1ra and/or TGFβ3 can be used to treat established PTOA* (Aim2a).

Specific Task is to test whether direct injection of the optimal dosage (determined in Specific Task 1 of Milestone 1) of genetically modified macrophages expressing IL-1ra and/or TGFβ3 into the synovium of the injured joint with established PTOA would improve the PTOA symptoms – Month 1 to Month 6.

Milestone 4 (Technical Objective 4). *To demonstrate that the macrophage-based IL-1ra and TGFβ3 combination adoptive gene therapy can effectively prevent development* of *PTOA* (Aim 2b).
Specific Task is to determine whether the adoptive therapy can be used to prevent development of PTOA – Month 7 to Month 12.

This annual progress report will summarize our progress from July 16, 2014 to February 28, 2015 and describe our progress towards Milestone 2.

Overall Project Aims

Milestone 2 Specific Task 1: To construct the required lenti-viral based vectors that can effectively express mouse IL-1ra, TGF-β3, or GFP marker genes

Progress: To generate mouse IL-1ra and TGF-β3 cDNAs, we purchased a mouse cDNA clone for each gene from Origene (Rockville, MD). We designed a pair of forward and reverse PCR primers that contain poly-A tails, AsisI or PMEI restriction enzyme site (forward: GCGATCGC; reverse: CAAATTTG), kozak sequence (GCCACCACC) and anti-sense sequence (15 to 30 bases). A unique DNA sequence of 527 bp (IL-1ra insert) and 1286 bp (TGF-β3 insert) was generated by PCR.

To subclone these two inserts into a pRRLsin-cPPT-SFFV-X-hPGK-GFP$_{wpre}$ plasmid (e.g., pSFFV-X), we first digested pRRLsin-cPPT-SFFV-cox2 -hPGK-GFP$_{wpre}$ plasmid (pSFFV-cox2) with restriction enzymes (e.g., AsisI and PMEI) to remove cox2. As shown in **Figure 1**, cox-2 (~2 kb) and pSFFV-X (~7.8 kb) are separated by the restriction enzyme digestion. Sticky ends on these two IL-1ra and

TGF-β3 inserts were made by AsisI/PMEI restriction enzyme digestion. To clone the inserts into pSFFV-X plasmid, we incubated insert and backbone in the presence of T4 ligase (Promega) at 16°C overnight. XL2-Blue Ultracompetent cells (Agilent Technologies) were infected with the ligation mix in the presence of antibiotics. Several individual colonies were randomly picked up and expanded in a LB medium at the presence of antibiotics. DNA was purified afterwards and positive clones were identified by restriction mapping. Specifically, we digested purified DNA with AsisI and PMEI and loaded digestion mix into an agarous gel. As shown in **Figure 2**, positive clones were identified by the presence of a unique size (527 bp for IL-1ra and 1286 bp for TGF-β3) of DNA fragments. To further verify the two positive clones, we digest them as well as pSFFV-cox2 plasmid with Kpni. As shown in **Figure 3**, the predicted size of DNA products (2427 and 3186 bp) are present, confirming the insertion of IL-1ra and TGF-β3.

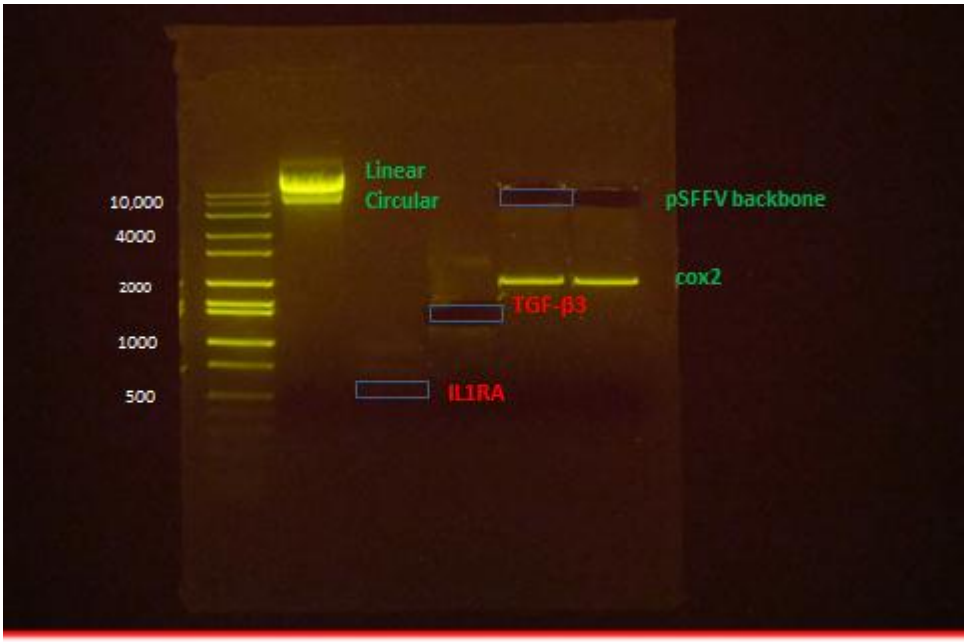

Figure 1. The image of the inserts of IL1-ra (lane 2), TGF-β3 (lane 3), and cox-2 (lanes 4 and 5) as well as the backbone of pSFFV-cox2 (lanes 4 and 5) after AsisI/pMEI restriction enzyme digestion. The gel bands containing IL-1ra (~527 bp), TGF-β3 (1286 bp), and pSFFV-X (7400 bp) are collected for subsequent ligation experiment. pSFFV-cox2 in a format of circular (without restriction enzyme digestion, lane 1) or of linear (one restriction enzyme; lane1) are used to determine the outcome of single restriction enzyme digestion and confirm the accuracy of electrophoresis.

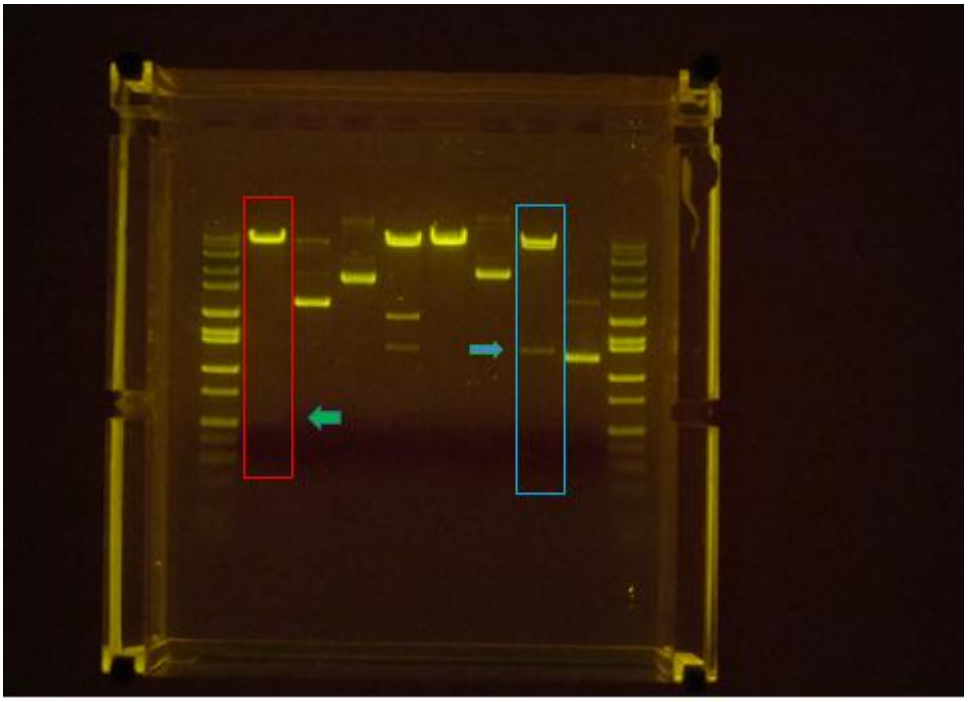

Figure 2. The image of positive clones for IL-1RA (red, lane 1, 527 bp) and TGF-β3 (blue, lane 7, ~1286 bp). Individual colony is randomly selected and expanded in LB medium at 37ºC for overnight. DNA was purified from each of expansion and digested with AsisI and pMEI. Positive clones are determined by the presence of 527 bp and 1286 bp for IL1-ra and TGF-β3, respectively.

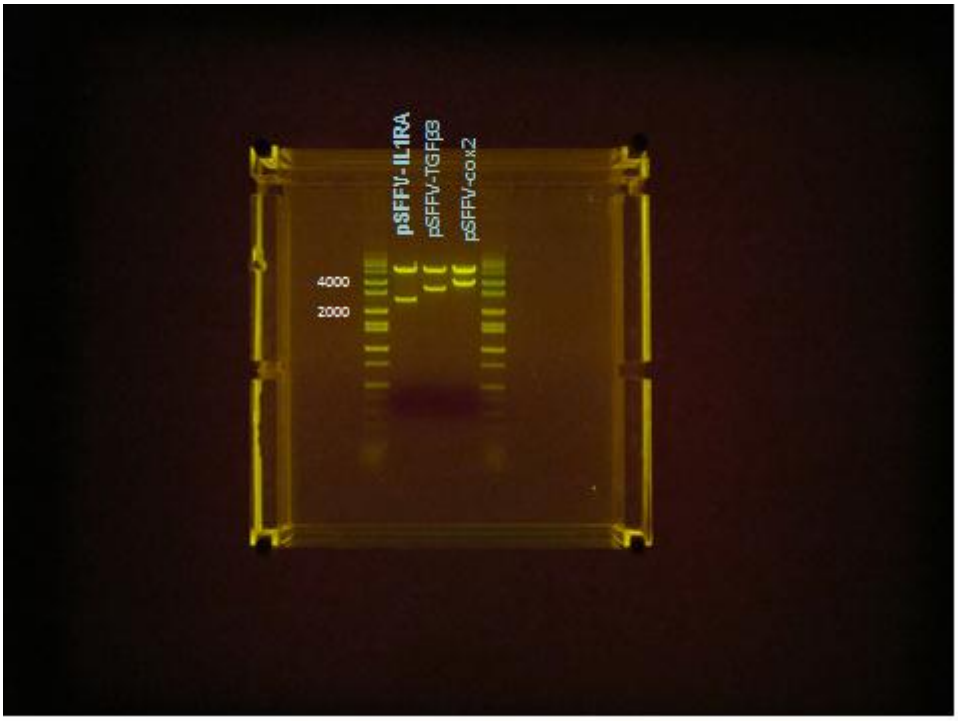

Figure 3. The image of DNA products derived from pSFFV-IL-1ra, pSFFV-TGF-β3, and pSFFV-cox2 plasmids by Kpni restriction enzyme digestion. The presence of 2427 and 5906 bp in lane 1 confirms positive for IL-1ra; The presence of 3186 and 5906 bp in lane 2 confirms positive for TGF-β3; The presence of 3811 and 5906 bp confirms positive for pSFFV-cox2.

Finally, pSFFV-IL-1ra and pSFFV-TGF-β3 were sent to McLab (South San Francisco, CA) for DNA sequencing. The sequencing results confirmed that two plasmids (e.g., pSFFV-IL-1RA and pSFFV-TGF-β3) contain the correct DNA sequence of the full length of IL-1RA and TGF-β3 cDNAs.

In sum, this specific task is 100% completed.

Milestone 2 Specific Task 2: To confirm that the isolated mouse macrophages can be effectively transduced by the lentiviral vectors expressing IL-1ra, TGFβ3, or GFP *ex vivo*, and to confirm that the transduced cells express substantial amounts of the transgene (Aim 1b)

Progress: To test whether macrophages can be effectively transduced with lentiviral particles containing IL-1ra or -TGF-β3, we first isolated bone marrow cells from adult C57BL/6J mice and cultured them for 3 days to eliminate attached cells. The unattached bone marrow cells were subsequently treated with mCSF for 3 days. These attached macrophages were then converted to M2 macrophages by IL-4. pSFFV-TGF-β3, IL-1ra, or pSFFV-GFP control (MOI=2 for each) viral vector was each added into M2 cultures ($5x10^5$ cells/well) in the presence of protamine sulfate (8 µg/ml) for 12 hours. Cultures were then replaced with fresh conditioned medium containing MCSF/IL-4 for additional 3 days. The conditioned medium was collected and frozen at -80ºC until ELISA assay is performed. Cell layers were extracted by 0.1% Triton x-100 for protein assay.

The production of TGF-β3 and IL-1ra was determined by commercially available ELISA assay kits (MyBiosource). As shown in **Figure 4**, M2 macrophages transduced with viral particles containing pSFFV-TGF-β3 exerted more than one-fold increase in TGF-β3 protein production in conditioned medium. This increase in TGF-β3 in conditioned medium was not due to a change in cell number as indicated by protein assay but to an increase in the production of TGF-β3 per se.

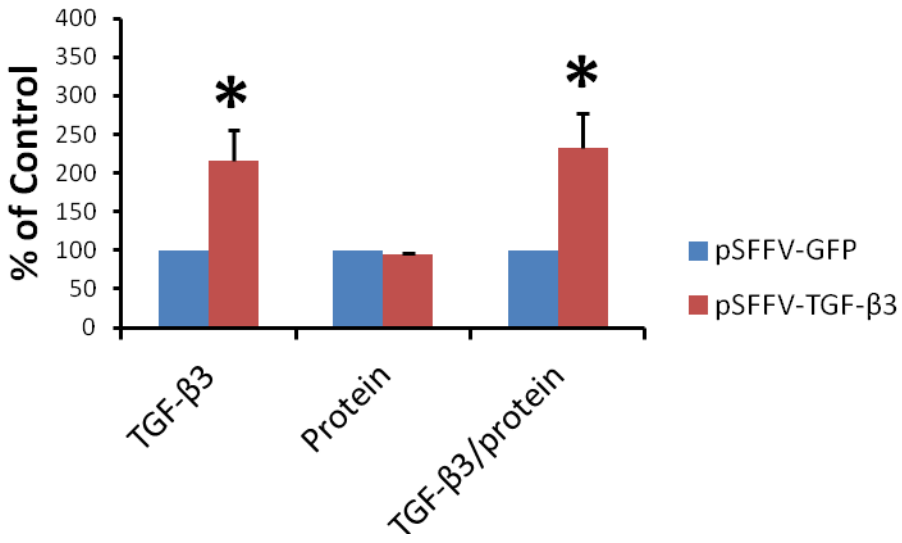

Figure 4. Comparison of the TGF-β3 production in conditioned medium of the cultures of macrophages expressing GFP control or TGF-β3 through lenti-viral based transduction. TGF-β3 is determined by commercial ELISA kit (MyBiosource). Cellular protein was determined by a BCA colorimetric assay (PIERCE). Three replicates per treatment. Data were presented as Mean±SD. * P<0.05 vs. pSFFV-GFP control.

In sum, this specific task is 100% completed.

IV. Key Research Accomplishments:

- We have successfully generate lenti-viral vectors expressing IL-1ra or TGFβ3 gene for use in the implantation experiment in next year of this project
- We have successfully shown that murine M2 macrophages can be transduced with lentirial vectors to express substantial amounts of IL-1ra or TGFβ3.
- We are in the process in testing the effect of injecting lentiviral vector transduced M2 macrophages into the PTOA knee joints to see if the treatment would reduce PTOA.

V. Conclusion

We have successfully accomplished most of Specific Tasks of Milestone 2. We are now in position to carrying the proposed work in Milestones 3 and 4, which is to access the clinical efficacy of the autologous macrophage-based anti-catabolic and pro-chondrogenic combination adoptive gene therapy for treatment and/or prevention of PTOA.

VI. Publication, abstracts, and presentation

There is none to report at this time.

VII. Inventions, patents, and licenses:

None.

VIII. Reportable Outcomes:

None.

IX. Other Achievement:

None.

X. References

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XI. Appendices:

None.